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Limited pollen flow and high selfing rates toward geographic range limit in an Atlantic forest bromeliad

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Bromeliaceae is a Neotropical family that evolved ecological key innovations in association with extensive adaptive radiation. Its species present a variety of different mating system strategies varying within and among species, within genera and subfamilies. Also, species with a wide geographical range can display large variation in mating system, reproductive success and genetic diversity. Here we combined data from hand pollinations and genetic analysis to assess outcomes of contemporary gene flow and mating system variation at the range edge of *Vriesea gigantea*. Results from pollen germination rates showed that this species is cryptically self-incompatible. Hand-pollination experiments and genetic analysis of progeny arrays revealed that *V. gigantea* has a mixed mating system, with high selfing rates ($s = 0.612$), and high inbreeding coefficient ($F = 0.372$). Inbreeding in *V. gigantea* at southern edge of its distribution range was caused by high levels of selfing rather than by mating among relatives. Moreover, strong pollen pool genetic structure was observed ($\Phi'_{FT} = 0.671$), with an increase from north to south. The parameters observed help us to understand historical and ecological conditions under which *V. gigantea* has experienced moderate to high levels of selfing in the face of reduced pollen flow from central to peripheral populations due to recent southward range expansion.

Introduction

Neotropics comprise the most species-rich region on Earth (Antonelli and Sanmartín, 2011). Nevertheless, relatively fewer genetic studies have examined Neotropical plant species, for example those species from the Atlantic Forest (AF), compared to temperate species (Hewitt, 2004; Jaramillo et al., 2006; Turchetto-Zolet et al., 2013). Bromeliaceae is an example of a Neotropical group that has evolved "key innovations" in association with its extensive adaptive radiation, like tank habit and crassulacean acid metabolism (CAM) (Givnish, 2010; Silvestro et al., 2014; Givnish et al., 2014). Moreover, it comprises subfamilies with different and relatively recent evolutionary histories. In particular, tank bromeliads from the AF have the highest diversification rate among bromeliad species, which can be associated with ecological trends such as evolution of differences in growth form, floral morphology, and mating systems (Benzing, 2000; Wendt et al., 2008; Givnish, 2010; Matallana et al., 2010; Givnish et al., 2011; Silvestro et al., 2014; Givnish et al., 2014). Studies on variation of reproductive traits within species can provide important insights in evolution and radiation of recent groups from Neotropics, especially in widespread species adapted to different ecological circumstances (Barrett, 2008 and references therein; Levin, 2012), and should be an important aspect for populations occurring at the species' distribution margins, where adaptations to specific conditions are decisive for their persistence (Darling et al., 2008; Geber, 2008; Sexton et al., 2009; Moeller et al., 2012).

Factors which determine species' range limits include abiotic and biotic factors, which are fundamentally related to the ecological niche of species (Sexton et al., 2009). A recent evolutionary

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model includes the notion that a fundamental parameter of mating system evolution, the inbreeding depression, may shape the association of traits along geographic distributions and create stable limits of edge dispersion if inbreeding depression decreases from center to periphery. This parameter is thus worth considering when analyzing empirical data (Pujol and Pannell, 2008; Cheptou and Massol, 2009; Pujol et al., 2009; Sun and Cheptou, 2012). Moreover, according to Baker's law, plants colonizing populations located at the margins of a distribution area tend to be autogamous as a consequence of pollinator or mate scarcity (Baker, 1955, 1959; Sun and Cheptou, 2012). Indeed, Kalisz et al. (2004), based on inbreeding depression and marker-based estimates of selfing for wild populations of *Collinsia verna* (Scrophulariaceae), demonstrated that when pollinator visits decrease, populations shift toward intermediate selfing rates through an increase in the proportion of autonomously selfed seeds. As widely known, selfing may allow reproduction when circumstances reduce opportunities for a union of gametes produced by different individuals, a phenomenon known as reproductive assurance (Holsinger, 2000).

Previous studies on reproductive biology of bromeliads showed mating strategies vary among species within genera and subfamilies (Wendt et al., 2001, 2002; Cascante-Marín et al., 2005; Barbará et al., 2007, 2008, 2009; Wendt et al., 2008; Matallana et al., 2010). Notwithstanding, amount of intraspecific variation in mating strategy and selfing capacity has only been assessed for a few species (Cascante-Marín et al., 2006; Paggi et al., 2007; Hmeljevski et al., 2011; Schmid et al., 2011; Zanella et al., 2011; Hmeljevski, 2013; Paggi et al., 2013). Yet such variation may have important consequences during colonization, as the establishment of selfing genotypes that may be favored after long-distance dispersal (the idea in Baker's law).

A species' mating system can be determined through hand-pollination experiments, progeny arrays and pollen pool genetic structure estimates. Also, biological characteristics and genetic patterns can help distinguish between potential mating (biological characteristics) and real mating (genetic patterns), assessing possible outcomes of gene flow at margins of a species' range (Holt and Keitt, 2005; Cheptou, 2012). In this study, the mating system and contemporary gene flow were reported for *Vriesea gigantea* Gaudich., a member of Tillandsioideae subfamily which exhibits high diversity in pollination modes and mating systems (Sazima et al., 1999; Benzing, 2000; Kessler and Krömer, 2000; Krömer et al., 2008; Wendt et al., 2008; Matallana et al., 2010). Mating systems of some Tillandsioideae species have been previously examined using non-molecular approaches, which have shown a particularly high frequency of selfing and mixed systems in *Alcantarea*, *Guzmania*, *Racinea*, *Tillandsia*, *Vriesea*, and *Werauhia* (Lasso and Ackerman, 2004; Ramírez-Morillo et al., 2004; Cascante-Marín et al., 2005; Paggi et al., 2007; Wendt et al., 2008; Ramírez-Morillo et al., 2009; Mattalana et al., 2010; Schmid et al., 2011; Paggi et al., 2013).

Furthermore, some previous reports on mating systems of bromeliads have been based on inferences from population genetic data (Wright's *F*-statistics) for a single plant generation. They have indicated mixed system for different species, *Alcantarea imperialis* (Carrière) Harms, *A. geniculata* (Wawra) J.R. Grant (Barbará et al., 2007, 2009), *A. glaziouana* (Lemaire) Leme and *A. regina* (Vell.) Harms (Barbará et al., 2008, 2009), *V. gigantea* (Palma-Silva et al., 2009), *V. minarum* (Baker) L.B.Sm. (Lavor et al., 2014) and outcrossing system for *Bromelia antiacantha* Bertol. (Zanella et al., 2011 see also Zanella et al., 2012 for a review), *Encholirium horridum* L.B.Sm. (Hmeljevski, 2013), and *Pitcairnia encholirioides* L.B.Sm., which is a self-incompatible species (Hmeljevski et al., 2014). Determination of mating system through a more comprehensive approach, using combinations of direct experiments and genetic analysis (e.g., progeny arrays), has been reported only for three bromeliad species, *Guzmania monostachia* (L.) Rusby ex Mez. and *Tillandsia fasciculata* Sw., both species showed high selfing rates (Cascante-Marín et al., 2006), and *Dyckia ibiramensis* Reitz, which presented mixed mating system (Hmeljevski et al., 2011).

In plants, mating systems have a profound effect on the genetic composition of natural populations (Hamrick, 1982). Likewise, variation in gene flow via pollen transfer and seed dispersal also contributes to the shape of genetic diversity within populations (Charlesworth, 2003; Duminil et al., 2007). Disruption of gene flow via pollen transfer and seed dispersal also contributes to loss of genetic diversity within populations isolated by habitat fragmentation (Hamrick, 1982), which may contribute to increase inbreeding coefficient. In a previous study, populations throughout entire *V. gigantea* (Bromeliaceae) distribution range showed latitudinal trends of decreasing genetic diversity and gene flow from north to south, which is consistent with historical forest expansion from northern half of the present distribution range (Palma-Silva et al., 2009). Further expansion toward the south appears to be inhibited by a lack of gene flow at edges of the current range (Palma-Silva et al., 2009; Paggi et al., 2010). Accordantly, Palma-Silva et al. (2009) found variation in the inbreeding coefficient (F_{IS}) among populations, with higher values occurring in *V. gigantea* populations from southern edge of range. In addition, Sampaio et al. (2012) also found geographic variation in inbreeding depression estimates, with a moderate value ($\delta = 0.31$), for the same central and southern populations of *V. gigantea*; those populations are the focus of the present study (Table 1). Herein, we investigate the mating system and contemporary gene flow of *Vriesea gigantea* at southern edge of the species range in Brazil, by combining data from hand-pollination experiments and genetic analysis of nuclear molecular markers. Specifically, we aimed to: a) characterize the mating system of *V. gigantea* at its southern range edge by estimating different parameters using progeny array and pollen pool genetic structure approaches, and by hand pollination experiments; and b) identify the role of two components, population subdivision or high selfing rate, that may be responsible for the high inbreeding coefficient (F_{IS}) observed previously. Results will help us to understand the relative roles of pollen flow and selfing in shaping southern range limit in *V. gigantea*.

Materials and methods

Study species

Vriesea gigantea (Tillandsioideae) presents an inflorescence with a central axis and several branches on each side, and flowers on each side of the lateral axis (Reitz, 1983; Fig. 1A). The flowers have three petals and their yellowish color and tube-shaped corolla are in accordance with the chiropterophilous syndrome (Vogel, 1969; Krömer et al., 2008). Besides, *V. gigantea* flowers lasted just one night (c.a. 12 h), and petals, stamens, and the style abscised during the following days. Also, the flowers present protogyny, in which style is receptive before anther dehiscence, in the beginning of anthesis (Paggi, 2009). The studied species is a tank bromeliad, frequently epiphytic or rupicolous, occurring in AF, across a wide geographic distribution, from Espírito Santo to Rio Grande do Sul states, Brazil (20°07′S, 40°30′W to 31°56′S, 52°25′W) (Fig. 1C). *Vriesea gigantea* is the only *Vriesea* species occurring in the southernmost part of AF (Smith and Downs, 1977; Reitz, 1983; Zimmermann et al., 2007; Martinelli et al., 2008; Palma-Silva et al., 2009; Fig. 1C).

Study sites

This study was conducted in three populations of *V. gigantea* (Maquiné, Itapuã and Taim; Table 1), located in AF of Rio Grande do

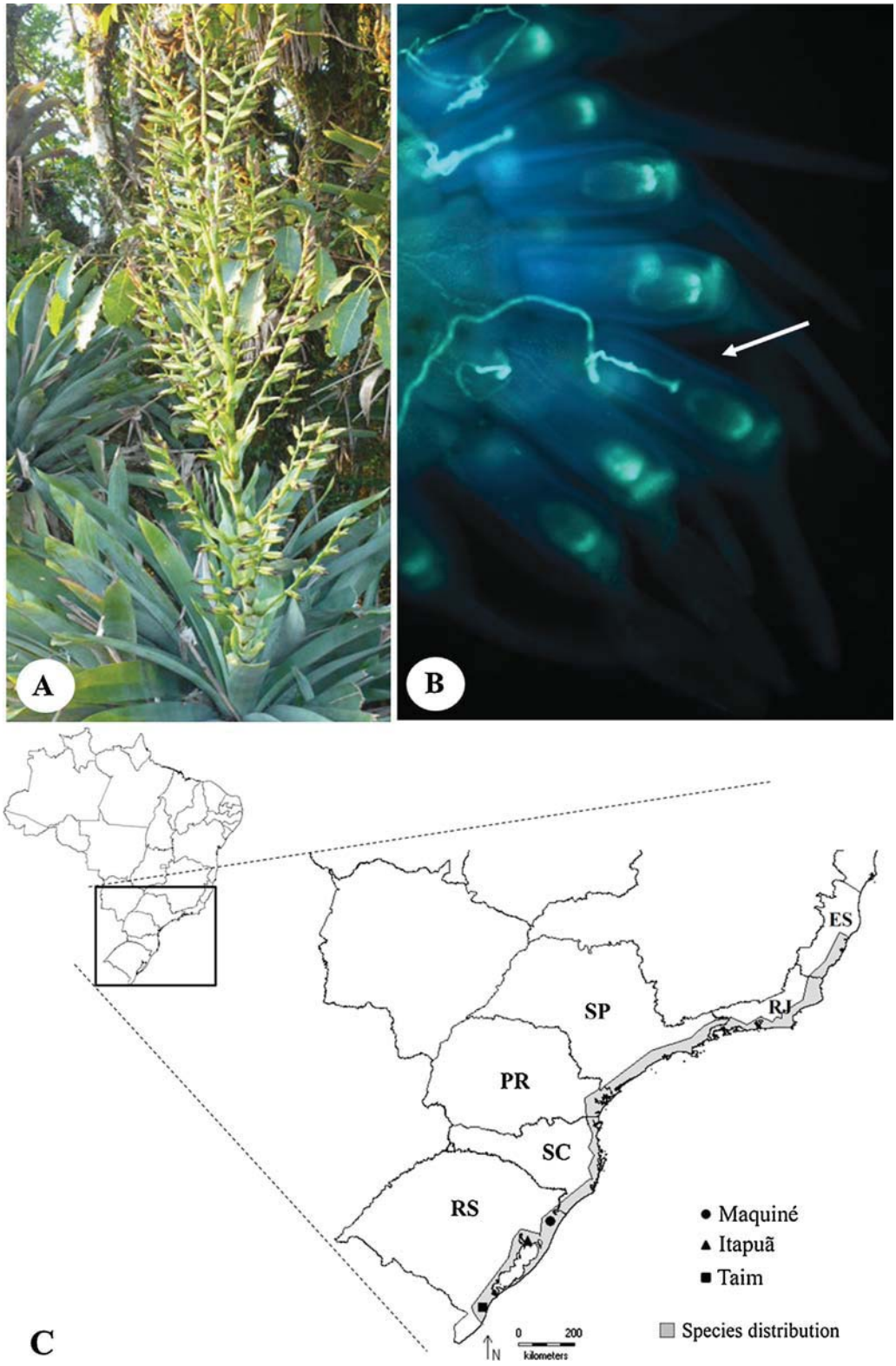

Fig. 1. (A) Individual showing large characteristic inflorescence of this species in the fruiting season. Photo: courtesy of CM Zanella. (B) Ovules showing pollen germination on a flower fixed 48 h after manual self-pollination, arrow showing a penetrated ovule. Photo: courtesy of N.S. Jr. Bittencourt. (C) Map showing the current geographic distribution of *V. gigantea* (Reitz 1983; Zimmermann et al., 2007; Palma-Silva et al., 2009) and the three studied sites from Rio Grande do Sul State, Brazil.

Sul state, Brazil. *Vriesea gigantea* is restricted to AF, which currently occurs in a narrow range in Brazilian coastal lowland, therefore we found single populations at different latitudes. Previous genetic analysis showed more central populations of Maquiné and Itapuã to have low gene flow with marginal population of Taim, the southernmost population (Palma-Silva et al., 2009). Climate in study region is classified as humid subtropical (Cfa) by the Köppen Climate Classification System. Fieldwork was carried out throughout the species' flowering and fruiting seasons (January–September 2006/2008). Itapuã population is included in a protected area of

Table 1
Natural populations of *V. gigantea* in Atlantic Forest, Rio Grande do Sul Brazil. Populations sampled with their geographical parameters, analysis performed, and plant habit.

Population	Conservation unit	City	Geographical coordinates	Habitat	Mean of temperature	Annual rainfall mm/year	*V. gigantea* habit	Mating system and pollen germination	Molecular analysis
Maquiné	Serra Geral ecological reserve	Maquiné	29°48′S, 50°16′W	Subtropical ombrophilous forest	21.5 °C	1650	Epiphytic/saxicolous	–	X
Itapuã	Itapuã state park	Viamão	30°22′S, 51°01′W	Sandy soil and shrubs with high light incidence	17.5 °C	1200	Epiphytic/saxicolous	X	X
Taim	Taim ecological station	Rio grande and Santa vitória do palmar	31°56′S, 52°25′W	Sandy coastal shrubs	18 °C	1100	Epiphytic exclusively	–	X

Table 2
Hand pollination experiments in *V. gigantea*: number of fruits and flowers used in each treatment. Fruit setting (%) obtained in each pollination treatment. Standard errors are in parentheses.

	Parameters	
Treatments	N° fruits/N° flowers	Fruit set (%)*
Control	22/47	47.9 (±9.6)a
Spontaneous selfing	13/48	27.1 (±6.3)b
Emasculated	5/35	17.7 (±8.8)b

* Estimates with the same letter are not significant different, ANOVA followed by the Tukey test (5%), $P = 0.0209$.

Itapuã State Park, created in the 1970's, which was previously affected by deforestation and stone extraction. However, there is a large number of specimens with more than 1000 adult plants distributed in clustered subpopulations in this population. Maquiné and Taim populations are located near the Ecological Reserve of Serra Geral and Taim Ecological Station, respectively, and have experienced some disturbance due to farming. In Maquiné population plants are distributed in clustered subpopulations, comprising more than 1000 adult individuals per subpopulation. In the southernmost population of Taim, specimens are distributed mainly on trees of *Ficus organensis* (Miq.) Miq., and in a lower density compared to other populations (personal observation).

Breeding system and pollen germination experiments

Hand-pollination experiments were performed in situ in Itapuã State Park population, where the experiment could be carried out without interference (Table 1). Controlled hand-pollination experiments were designed to test for spontaneous selfing (bagging) and outcrossing (in emasculated open flowers). Flowers under natural pollination conditions were used as controls. In emasculation process, anthers were removed gently before anthesis; in bagging process, mesh bags were used (maximum mesh size 0.9 mm) to cover the flowers. Three to five flowers were used per plant, in a total of 16 plants, totaling 47 flowers for the control, 48 for the spontaneous selfing, and 35 for the outcrossing treatments (Table 2). All treatments were applied in all sampled individual plants. Fruit set (i.e., the fraction of flowers that developed into mature fruit) was estimated as % fruits = number of fruits per plant/number of flowers per plant × 100, for sampled flowers per plant in each treatment. Differences in fruit set among treatments was detected using ANOVA followed by Tukey test (5%) (SAS, version 8).

In the same population, manual self- and cross-pollination treatments were performed to compare differences in pollen tube growth and number of penetrated and non-penetrated ovules. Experiments were performed at beginning of anthesis, when stigmas were receptive, and before anther dehiscence (Paggi, 2009). After 48, 72, or 96 h, flowers from hand self- and cross-pollination were fixed in 3:1 ethanol: glacial acetic acid, to allow further observation using fluorescence microscopy and aniline blue staining (Martin, 1959). Five to seven pistils per individual per treatment were prepared for examination, for a total of 33 pistils. Proportion of penetrated ovules in the self- and cross-pollinated pistils was assessed by scraping out ovules and scoring for presence of pollen tube "tails" at micropyle. Differences in the mean proportion of penetrated ovules in the self- and cross-pollinated pistils were detected using *t*-test (SAS, version 8).

Mating system analysis

Naturally pollinated maternal seeds were randomly collected from all available reproductive individuals at all studied

populations, five mother plants (MPs) in Taim, 10 in Maquiné, and 11 in Itapuã, for a total of 26 MPs. Location of each MP was recorded using a global positioning system (GPS) receiver for TwoGener analysis. On average, 17 seedlings from each MP were used for genetic analysis, for a total of 501 progenies. The seedlings were obtained by *in vitro* seed germination, as described by Paggi et al. (2007). Fresh leaves (~5 cm^2) from MPs and seedlings were stored in liquid nitrogen until DNA extraction. Total genomic DNA was extracted using the protocol described by Doyle and Doyle (1990).

Eight microsatellite loci previously characterized for bromeliad species *Tillandsia fasciculata*, *Guzmania monostachia* (loci: e6b and CT5, respectively; Boneh et al., 2003), and *V. gigantea* (loci: VgA04, VgA06, VgB06, VgF01, VgF02, and VgG02; Palma-Silva et al., 2007) were selected for this study considering their appropriated amplification in study species and polymorphism observed in each sampled population. We performed PCR reactions in a PE Applied Biosystems 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) as described by Palma-Silva et al. (2007). Microsatellite alleles were resolved on a 3100 DNA Analyzer (Applied Biosystems) and precisely sized against a ROX molecular size standard (Applied Biosystems) using Genescan 3.7 and Genotyper 3.7 (Applied Biosystems).

Multilocus outcrossing rate (t_m) was estimated under mixed mating model described by Ritland (2002), with Mltr 3.4 (Ritland, 2009). This procedure allowed us to estimate multilocus outcrossing rate (t_m), mean single-locus outcrossing rate (t_s), difference between estimates (t_m–t_s), which represents the outcrossing rate between related individuals (=biparental inbreeding), and inbreeding coefficient of maternal parents (F). Significance of t_m and F were determined using one-tailed Student's t-tests based on null hypotheses that t_m is significantly less than 1 and F is significantly greater than zero (SAS, version 8). Mltr software also estimates correlated mating parameters: correlation of selfing (r_s), which reflects variation in selfing rates among families, and the correlation of paternity (r_p), which is the probability that two siblings are outcrossed full-sibs (Ritland, 2002). Significance of r_s was also determined using one-tailed Student's t-tests based on null hypotheses that r_s is significantly greater than zero (SAS, version 8). Standard errors were estimated based on 1000 bootstraps between individuals within a progeny array. From these estimates, we calculated the proportions of progeny that are full sibs ($t_m \cdot r_p$) and half sibs [$(t_m\ (1-r_p)$] (Mantovani et al., 2006).

TwoGener procedure was used to analyze genetic structure of pollen pools (Φ_{FT}) of individual plants relative to global pollen pool (Smouse et al., 2001) based on AMOVA approach of Excoffier et al. (1992). We computed 99% confidence interval of Φ_{FT} by bootstrapping among loci with 1000 replicates, using Poldisp 1.0 software (Robledo-Arnuncio et al., 2007). Significance of Φ_{FT} was determined using one-tailed Student's t-tests based on null hypotheses that Φ_{FT} is significantly less than 1 (SAS, version 8).

Inbreeding coefficient was high and significant in adult populations of *V. gigantea* (F_{IS} = 0.269; Palma-Silva et al., 2009), which may bias estimates of pollen pool genetic structure, increasing this estimate. Hence, the estimate of Φ_{FT} was corrected using the formula described by Austerlitz and Smouse (2001), $\Phi'_{FT} = \Phi_{FT}(1+F)$, where Φ_{FT} is pollen structure parameter due only to limited pollen dispersal, and F is adult inbreeding coefficient. Estimate of Φ'_{FT} was used to calculate variance in pollen flow, computed as $\sigma^2 = (\Phi_{FT} 8\pi d)^{-1}$, average distance of pollen dispersal ($\delta = \sqrt{\sigma^2 \pi}/2$), effective neighborhood pollination area ($A_{ep} = 4\pi\ \sigma^2$), and effective number of pollen donors ($N_{ep} = 4\pi\ \sigma^2 d$) (Austerlitz and Smouse, 2001). ANOVA followed by Tukey test were performed to determine whether genetic parameters differed among localities using families as grouping factors. All statistical analyses were performed using SAS (version 8).

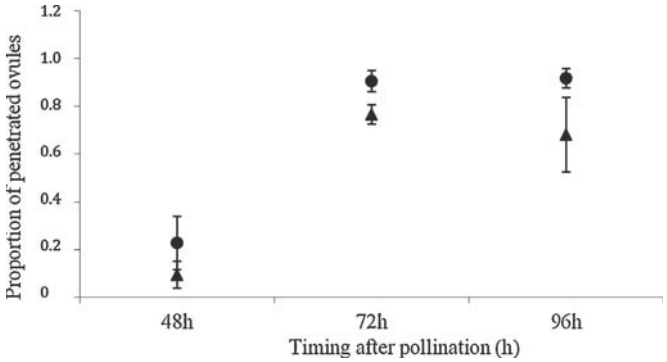

Fig. 2. Proportion of penetrated ovules in 48, 72 and 96 h after self (triangles) and cross-pollination (circles).

Results

Breeding system and pollen germination experiments

Results from hand-pollination experiments showed that proportion of fruit set was 47.9% in control treatment, 27.1% in spontaneous selfing treatment, and 17.7% in emasculated treatment, which estimates the amount of pollen from other flower (from same or other individual) in the emasculated flowers (Table 2). Fluorescence microscopy analysis showed both self- and cross-pollinated grains germinated on stigma and produced tubes that grew toward ovary (Figs. 1B, 2). A higher proportion of penetrated ovules were observed in cross-pollinated flowers than self-pollinated at every time points tested. At 48 h after self-pollination and cross-pollination, 9.5% and 22.8% of ovules, respectively, were penetrated (F = 5.63, P = 0.0451). After 72 h, around 90% of ovules were penetrated in the cross-pollination treatment, while 76% of ovules were penetrated in self-pollination treatment (F = 33.47, P = 0.0001). Difference was even greater after 96 h, with 92% of cross-pollinated ovules penetrated and 68% of self-pollinated ovules penetrated (F = 10.69, P = 0.0114; Fig. 2). Pollen tubes from self-fertilization grew more slowly than pollen tubes from cross-fertilization; as a result, cross-pollination occurs foremost in flowers of *V. gigantea*.

Mating system and contemporary gene flow

Outcrossing rate (t_m) was variable among populations, ranging from 0.176 to 0.477; outcrossing rate across all populations was 0.388 (Table 3). A significant self-fertilization rate ($s = 1 - t_m$) was detected (s = 0.612) since the overall outcrossing rate was significantly lower than one (t = – 22.778, P < 0.0001). Outcrossing rate between relatives (biparental inbreeding; t_m–t_s) ranged from 0.069 to 0.141, south to center, respectively. Estimates for individual MPs showed significant correlation in selfing (r_s, among-plant variation in the selfing rate, t = – 17.129, P < 0.0001) within the Maquiné population alone. Distribution of individual outcrossing rates for this population indicated correlation probably arose from few families exhibiting significant selfing (s > 0.20; data not shown).

Inbreeding coefficient (F), which is based on genotypic frequencies in maternal plants, was positive and significantly greater than zero (F = 0.372; t = – 12.524, P < 0.0001; Table 3), considering all populations. Maquiné and Itapuã populations had higher outcrossing rates (0.477 and 0.336, respectively) compared to Taim population, which showed the highest selfing rate (0.824). Mating system parameters exhibited conspicuous trends toward southern margin of species' range, with some parameters increasing and others decreasing (Tables 3 and 4). Multi- and single-locus outcrossing rates and correlation of paternity showed significant differences

Table 3
Estimates of mating system parameters (SE in parentheses) for three *V. gigantea* populations from Rio Grande do Sul State, Brazil. * Mixed mating system parameters: *F*, (estimated) parental inbreeding; t_m, multilocus outcrossing rate; t_s, single-locus average outcrossing rate; ($t_m - t_s$), biparental inbreeding; r_p, correlation of paternity; r_s, correlation of selfing; and N_{ep}, effective number of pollen donor.

	Parameter	Maquiné	Itapuã	Taim	All
Mixed mating system model	F	0.212 (0.011)	0.355 (0.008)	0.718 (0.012)	0.372 (0.004)**
	t_m*	0.477 (0.028) a	0.336 (0.029)ab	0.176 (0.033)b	0.388 (0.017)**
	t_s*	0.337 (0.022) a	0.225 (0.021)a	0.106 (0.020) b	0.244 (0.011)
	($t_m - t_s$)	0.141 (0.018)	0.111 (0.020)	0.069 (0.015)	0.144 (0.012)
	r_p	0.714 (0.070)	0.476 (0.093)	0.386 (0.395)	0.631 (0.044)
	r_s*	0.603 (0.083)*** a	0.121 (0.096)b	−0.021 (0.481)b	-0.200 (0.014)
	N_{ep}	1.4	2.1	2.6	1.58
	Full sibs ($t_m \cdot r_p$)	0.341	0.159	0.068	0.245
	Half sibs [(t_m (1 − r_p)]	0.136	0.176	0.108	0.143

* Estimates with the same letter are not significant different, ANOVA followed by the Tukey test (5%). Correlation of selfing r_s, ($F = 8.92$; $P = 0.0024$); multilocus outcrossing rate t_m, ($F = 5.69$; $P = 0.0106$); and single-locus average outcrossing rate t_s, ($F = 7.06$; $P = 0.0036$).
** One-tailed Student's *t*-tests: $F > 0$, $t = -12.524$, $P < 0.0001$; $t_m < 1$, $t = -22.778$, $P < 0.0001$; $r_s > 0$, $t = -17.129$, $P < 0.0001$.

Table 4
Estimates of pollen pool genetic structure parameters based on TwoGener Model for three *V. gigantea* populations from Rio Grande do Sul State, Brazil. TwoGener model parameters: Φ'_{FT}, corrected pollen structure parameter; Φ_{FT}, observed (uncorrected for inbreeding) pollen structure parameter; N_{ep}, effective number of pollen donors; δ, average distance of pollination; and A_{ep}, effective neighborhood pollination area.

Population/ Parameter	Maquiné	Itapuã	Taim	All
Φ'_{FT}*	0.491	0.768	0.928	0.671***
Φ_{FT}	0.401	0.516	0.659	0.532
N_{ep}*	1.02	0.65	0.54	0.75
δ (m)	137	403	245	147
A_{ep} (m^2)	150	1302	480	173

* Parameters not significantly different among populations. ANOVA followed by the Tukey test (5%). ($F = 1.12$; $P = 0.3450$).
*** One-tailed Student's *t*-tests: $\Phi'_{FT} < 1$, $t = -24.164$, $P < 0.0001$.

among populations, being lower in southernmost population of Taim (Table 3).

Genetic structure of pollen pool in *V. gigantea* populations was $\Phi_{FT} = 0.532$, indicating significant differentiation in pollen pools among populations (Table 4). Considering significant inbreeding coefficients previously observed in a "single-generation" population genetic study ($F_{IS} = 0.269$; Palma-Silva et al., 2009), we corrected pollen pool structure parameter and found a slightly greater value of $\Phi_{FT} = 0.671$ ($t = -24.164$, $P < 0.0001$; Table 4). From this estimate ($\Phi_{FT} = 0.671$), effective number of pollen donors (N_{ep}) was calculated as 0.75, effective neighborhood pollination area (A_{ep}) was 173 m^2, and average distance of pollination (δ) was 147 m, considering all populations. In agreement with Ritland's mating system parameters which decreased from north to south, Φ_{FT} values increased toward species' range edge (Table 4).

Discussion

Combining results from hand-pollinations experiments, pollen germination and progeny array analysis, we showed that *V. gigantea* exhibits mixed mating system and cryptic self-incompatibility, consistent with levels of genetic diversity data reported by Palma-Silva et al. (2009). Population in extreme southern edge of the species' distribution (Taim) exhibited the highest selfing rates and pollen pool genetic structure. Results presented here are important for ongoing debates regarding limits to adaptation in marginal plant populations, particularly considering variation and evolution of plant mating systems. Ecological and genetic patterns play important roles in persistence of plant populations in marginal portions of their geographic distribution (Pannell and Dorken, 2006; Eckert et al., 2008; Geber, 2008; Pujol and Pannell, 2008; Foxe et al., 2009; Pujol et al., 2009; Barringer et al., 2012).

Mixed mating system and cryptic self-incompatibility

Based on results from hand-pollination experiments and pollen germination, *V. gigantea* exhibits a mixed mating system, since it can set fruits through both self-fertilization and outcrossing (Table 2; Paggi et al., 2007). Mixed mating systems are particularly common in self-compatible species pollinated by animals (Barrett and Eckert, 1990; Barrett and Charlesworth, 1991; Vogler and Kalisz, 2001; Barrett, 2002, 2003; Goodwillie et al., 2005; Futuyma, 2009; Goodwillie et al., 2010). Moreover, selfing component of a mixed mating system could arise through geitonogamy (G.M. Paggi and C. Palma-Silva, personal observations) and might be a non-adaptive cost associated with the large floral display of *V. gigantea* (Reitz, 1983; Sazima et al., 1999; Paggi et al., 2007), which is typically required to attract animal pollinators. Sazima et al. (1999), based on floral features and pollinator observations in populations from southeastern Brazil, concluded *V. gigantea* is an outcrossing and bat-pollinated species; nevertheless, that study was performed only at the center of the species' distribution (southeastern Brazil, 23°50′S, 46°08′W), and the present study was conducted at the central-southern edge of the species' range (Table 1). The geographic distribution of the bat species described as pollinator of *V. gigantea* in southeastern Brazil, *Anoura caudifer* É. Geoffroy, only reaches Maquiné population in southern Brazil; this bat species does not occur in Itapuã or Taim populations (Fábian et al., 1999; Oprea et al., 2009). In addition, we observed populations from southern Brazil have diurnal anthesis and are preferentially pollinated by bees and hummingbirds rather than bats (Paggi, 2009). These facts indicate a shift in reproductive biology of *V. gigantea* across its geographic distribution. Populations at margin of a species' distribution may experience poorer environmental conditions than populations in center of range (Geber, 2008). Therefore, marginal populations may have low fitness (Tsaliki and Diekmann, 2009; Barringer et al., 2012) or may face reductions in pollinator services resulting in higher cross-pollination limitation, which would consequently lead to high selfing rates through autonomous selfing, the reproductive assurance (Holsinger, 2000; Barrett, 2002; Darling et al., 2008; Zhang and Li, 2008; Cheptou, 2012; Moeller et al., 2012). Surprisingly, based on flower, fruit and seed production (Paggi et al., 2007; Sampaio et al., 2012) and adult morphology aspects, seed germination and seedling survival, high levels of plant fitness for *V. gigantea* populations at the range margin were reported (Sampaio et al., 2012), although pollen limitation was observed (Paggi et al., 2007; Palma-Silva et al., 2008).

Pollen germination experiments revealed that *V. gigantea* is self-compatible as described by (Paggi et al., 2007), but presents cryptic self-incompatibility, which is a form of physiological SI found in self-compatible plants in which outcrossing is promoted through differential pollen-tube growth between outcross- and self-pollen

tubes (Franklin-Tong, 2008). Several plant species exhibit cryptic forms of self-incompatibility in which seed set following cross- and self-pollination is similar but differences exist in germination speed and quality, suggesting that some form of competition exists between self- and cross-pollination (Good-Avila et al., 2008). Self-compatibility appears to be a widespread trait in Bromeliaceae, mainly in the subfamily Tillandsioideae, including species from *Alcantarea*, *Guzmania*, *Racinea*, *Tillandsia*, *Vriesea*, and *Werauhia* (Lasso and Ackerman, 2004; Ramírez-Morillo et al., 2004; Cascante-Marín et al., 2005, 2006; Matallana et al., 2010; Schmid et al., 2011). Growing evidence indicates many self-compatible plants control the level of self-fertilization through post-pollination processes that give a siring advantage to cross-pollination over self-pollination through cryptic SI (Eckert and Allen, 1997). This mechanism has also been reported in several species of Lythraceae, Melastomataceae and Spigeliaceae as CSI and may function to reduce inbreeding (Eckert and Allen, 1997; Erbar and Leins, 1999; Erbar, 2003; Santos et al., 2010). Paggi et al. (2013) reported self-sterility due to late-acting SI in a congener species, *V. friburgensis* Mez, another study conducted in a species' range edge. In the present work, selection for reduced CSI may have occurred at periphery of species' range, but this has not been tested nonetheless.

High selfing rates and inbreeding coefficient

Results from the progeny array showed moderate to low outcrossing rates in populations of *V. gigantea* (Table 3), which agrees with mixed mating system. High inbreeding coefficient observed ($F = 0.212–0.718$) was similar to previous results from same populations in AF ($F_{IS} = 0.225–0.489$; Palma-Silva et al., 2009). This result can be explained by high levels of selfing ($s = 0.612$) rather than mating among relatives ($t_m – t_s = 0.144$) in studied populations (Table 3). Since in *V. gigantea* each plant opens many concurrently flowers per day (Sazima et al., 1999 G.M. Paggi personal observation), observed low outcrossing, high inbreeding coefficient and high levels of selfing are probably due to pollinator-mediated autogamy and geitonogamy, apparently contributing to high selfing rates in *V. gigantea*. Also, a positive value of r_s was observed in Maquiné population, which means that two selfed progeny sampled from same progeny array are not result of two independent selfing events. For instance, such correlated selfing may occur when movement of a pollinator causes within-flower or -individual selfing (G.M. Paggi personal observation). Therefore such correlations are expected to be high among seeds within a single fruit (Takebayashi et al., 2006). Likewise, correlation of paternity (the probability of two siblings being outcrossed full-sibs) varied among populations, and was high in Maquiné, where outcrossing rate was also high, indicating that even with pollinator service, gene flow persist low, with few pollen donors per population and among populations (Table 3).

TwoGener analysis indicated significant differentiation in pollen pool genetic structure among populations, with pollen-mediated gene movement being severely limited ($\Phi'_{FT} = 0.671$; Table 4). Effective pollination neighborhood (A_{ep}) around a MP was only 173 m^2, suggesting that MPs are preferentially pollinated by pollen drawn from neighboring plants (average distance of pollination $\delta = 147$ m; Table 4). This was confirmed by moderate level of biparental inbreeding ($t_m – t_s = 0.144$). Likewise, *V. gigantea* has low gene flow via seeds, since direct observations showed seeds disperse over short distances (<3 meters, Paggi et al., 2010). Moreover, low effective numbers of pollen donors (N_{ep}; Tables 3 and 4) indicate that the majority of seeds within each fruit were sired only by a few pollen donors, with few progeny having different fathers, either due to pollen carryover or multiple pollinator visits.

Limited pollen flow toward geographic range limit

Outcrossing rates and pollen pool genetic parameters showed a characteristic trend, with increased selfing and pollen pool genetic structure toward southern margin of species' range, from Maquiné (central) to Taim (southern) populations (Tables 3 and 4). At southern range edge, Taim population, it was observed low levels of outcrossing accompanied by a strong pollen pool genetic structure. As a result, studied populations may differ in their effectiveness at purging deleterious alleles; i.e., recessive deleterious alleles may have been exposed and purged more efficiently in Taim population due to selfing (Futuyma, 2009; Barringer et al., 2012). Variation in mating systems among populations (intra-specific) and species (inter-specific) has been documented in many plant species, including bromeliads (Cascante-Marín et al., 2006; Barbará et al., 2007, 2009), and the most common factors put forward to explain the observed variation are historic range changes (Sun and Ritland, 1998; Cascante-Marín et al., 2006; Geber, 2008; Pujol et al., 2009; Barringer et al., 2012), habitat fragmentation (Ohara et al., 2006; Hmeljevski et al., 2011), and reproductive isolating mechanisms (considering the evolution of selfing; Wendt et al., 2001, 2002; Barbará et al., 2007; Wendt et al., 2008; Barbará et al., 2009; Matallana et al., 2010; Palma-Silva et al., 2011).

Considering populations of *V. gigantea* sampled throughout its geographic distribution, Palma-Silva et al. (2009) reported a strong negative correlation between genetic diversity and population latitude, consistent with historical Atlantic Forest expansion from northern half of present distribution range (Rambo, 1951, 1960; Smith, 1962; Turchetto-Zolet et al., 2013). Generally, populations at edges of species' ranges may face higher risks of extinction if they are less able to respond to selection under a changing environment (Pannell and Dorken, 2006; Eckert et al., 2008; Geber, 2008; Pujol and Pannell, 2008; Levin, 2012). Also, two major factors, the timing and a severe founder event (bottleneck), support hypothesis that selfing may have been favored during colonization as new habitats emerged after the last glaciation (Pannell and Dorken, 2006; Foxe et al., 2009; Pujol et al., 2009). Studying populations of *V. gigantea* from the range margin, Sampaio et al. (2012) reported low to moderate levels of inbreeding depression ($\delta = 0.02$ to 0.39), which was consistent with our previous results on fertility, genetic diversity and gene flow (Paggi et al., 2007; Palma-Silva et al., 2009). Besides, Paggi et al. (2010) described short distances of seed dispersal for this species, in agreement with reported aggregate distribution of bromeliad seedlings (Zotz, 1997; van Dunné, 2001; Cascante-Marín et al., 2005), and with previous molecular studies, revealing *V. gigantea* populations are genetically structured, with low levels of gene flow (Palma-Silva et al., 2009 this study). All analyzed parameters indicates that historical and ecological conditions following the Pleistocene climatic oscillations are reflected in the contemporary genetic structure of *V. gigantea*, which has experienced reduction in gene flow (Palma-Silva et al., 2009 this study) and low to moderate levels of inbreeding toward the southern margin of its distribution (Sampaio et al., 2012).

Limited pollen flow and high selfing rates observed in the marginal population (Taim) of *V. gigantea* could have been shaped by a recent southward expansion (founder effect), together with a decrease in inbreeding depression (Baker, 1955, 1959; Herlihy and Eckert, 2005; Cheptou and Massol, 2009; Barringer et al., 2012; Sampaio et al., 2012; Sun and Cheptou, 2012), contributing to species adaptation to abiotic and biotic factors at range limits (Sexton et al., 2009; Vaupel and Matthies, 2012). This prediction is supported by many examples of plant groups in which selfing taxa or populations occur in areas that are geographically or ecologically marginal relative to their related outcrossers (Schoen et al., 1996; Barrett, 2002; Arnaud-Haond et al., 2007; Mimura and Aitken, 2007; Darling et al., 2008; Barringer et al., 2012). Previous

results have shown that marginal population (Taim) of *V. gigantea* presents low levels of genetic diversity (Palma-Silva et al., 2009), which can be a result of colonization bottlenecks affecting local adaptation after range expansions (Eckert et al., 2008; Geber, 2008; Pujol and Pannell, 2008; Excoffier et al., 2009). However, Taim population maintains high fitness, with high production of flowers, fruits and seeds, high germination rates (Sampaio et al., 2012) and high pollen viability (Palma-Silva et al., 2008) with low inbreeding depression (Sampaio et al., 2012), thus suggesting *V. gigantea* has great potential to adapt to increased levels of selfing at the southern range limit.

Final remarks

CSI of *V. gigantea* favors germination and fertilization of outcross pollen grains over selfing ones, which results in a mixed mating system in this self-compatible species. Those features, combined with protogyny and floral display, may contribute to mixed mating system, since they promote delayed selfing and represent a trade-off between pollinator attraction and geitonogamous selfing. CSI may also contribute to the maintenance of high levels of genetic diversity and fitness and moderate levels of inbreeding depression (Paggi et al., 2007; Palma-Silva et al., 2008, 2009; Sampaio et al., 2012 this study), which could facilitate the persistence of this species at its range edge. High selfing rates observed in the studied populations were the main processes that created high F_{IS} values in the southern range margin. However, population subdivision due to biparental inbreeding (Table 3) could also contribute to elevated F_{IS} values in populations at southern edge, since low levels of pollen flow (high estimates of pollen pool genetic structure) were observed in all populations.

Although number of investigated families and populations is limited, our results support the idea that selfing ability associated with a decrease in inbreeding depression (Sampaio et al., 2012) from center to periphery can have great importance for plant population establishment at stable range limits (Sun and Cheptou, 2012). This is in line with results for other plant groups and helps to explain the evolution of selfing in plant species (Cheptou et al., 2002; Darling et al., 2008; Geber, 2008; Pujol et al., 2009; Barringer et al., 2012; de Groot et al., 2012). A solid knowledge base of genetic variation and reproductive biology in bromeliad species with different mating systems and ecological adaptations would provide key information for better understanding the importance of shifts in mating systems during diversification of Bromeliaceae. This information would also benefit studies that assess impacts of habitat fragmentation on biological diversity in this large ecologically important plant family that has undergone continent-wide adaptive radiation.

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